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An important role of the heat shock response in infected cells for replication of baculoviruses

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ABSTRACT

Baculoviruses serve as a stress factor that can activate both death-inducing and cytoprotective pathways in infected cells. In this report, induction of heat shock proteins (HSPs) of the 70-kDa family (HSP/HSC70) in Sf-9 cells after infection with AcMNPV was monitored by Western blot analysis. Two-dimensional electrophoresis in polyacrylamide gel revealed changes in the cellular pattern of HSP/HSC70s and synthesis of a new member of the HSP/HSC70 family in the infected cells. Although infection with AcMNPV moderately increased the HSP/HSC70 content in cells under standard conditions, the infection potentiated the response to heat shock boosting the HSP/HSC70s content in infected cells several-fold in comparison with uninfected cells. Addition of KNK437, a known inhibitor of inducible HSPs, decreased the rate of viral DNA synthesis in infected cells more than one order of magnitude and markedly suppressed the release of budded viruses indicating the importance of the heat shock response for baculovirus replication.

Introduction

Baculoviruses contain circular double-stranded DNA genomes of 80 to 180 kb and belong to the family *Baculoviridae*, and different members are infectious predominantly for insects of the orders Lepidoptera, Hymenoptera, and Diptera. Replication of viral DNA (vDNA) and the assembly of viral capsids occur in cell nuclei and yield two types of mature viruses, the budded virions (BV) and occlusion-derived virions (ODV). *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is the best-studied baculovirus and is widely used for the generation of recombinant vectors for the expression of foreign genes. The 134-kbp genome of AcMNPV encodes about 150 proteins including six factors essential for vDNA synthesis in transient assays including the transcriptional activator IE-1, DNA helicase, DNA polymerase, DNA primase (LEF-1), primase-associated factor (LEF-2), and a single-stranded DNA-binding protein (SSB) protein (LEF-3) (for review see (Rohrmann, 2008)). Although baculoviruses encode key factors required for genome replication, they likely employ the host-cell systems for the synthesis and processing of their proteins. In eukaryotic cells, posttranslational folding of proteins, intracellular trafficking, and quality control are performed by molecular chaperones, abundant heat shock proteins (HSPs). These proteins are numerous and highly diverse and may include up to hundred different species that range from 12 to 110 kDa (for review see (Feder and Hofmann, 1999)). The ubiquitous chaperone family of the 70-kDa HSPs and cognate heat shock proteins (HSC70) plays a central role in protein homeostasis and protection against proteotoxic stresses by preventing protein misfolding and aggregation, or by directing damaged proteins to the ubiquitin-proteasome system for degradation. The ATP-dependent chaperoning activity of HSP/HSC70s is regulated by a battery of co-chaperones, which specify the function of HSPs and regulate their interaction with client proteins. Some members of the HSP/HSC70 family are constitutively expressed in cells, whereas others are induced by a wide variety of physiological and environmental insults including heat, reactive oxygen species, or drugs under pro-survival pathway named heat shock response (HSR). A major role in activation of this pathway is played by transcription factor HSF (HSF1 in vertebrates). Increasing data indicate that inducible HSPs are involved in the replicative cycles of various viruses including adenoviruses (Ads), polyomaviruses (PyVs), human papillomaviruses (HPVs), herpesviruses, and some RNA viruses (Chen et al., 2009; Dutta et al., 2009; Lahaye et al., 2009; Livingston et al., 2009; Mayer, 2005; Weeks et al., 2010; Zhao et al., 2009). HSP70s regulate gene expression by interacting with viral proteins and participate in capsid assembly and disassembly for PyVs, HPVs, and some RNA viruses (Couturier et al., 2010; Hu et al., 1997;

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Mayer, 2005; Song et al., 2010). Chaperone-enriched domains are formed in the nuclei of cells undergoing lytic infection with herpes simplex virus type 1 (HSV-1). These virus-induced chaperone-enriched (VICE) domains, contain HSC70, HSP70, HSP40, HSP90, poly-ubiquitinated proteins, and components of the proteasome machinery (Burch and Weller, 2004, 2005; Livingston et al., 2008; Livingston et al., 2009; Mathew et al., 2009). Accumulating evidence indicates that these sites may be utilized during infection to sequester misfolded, modified, or otherwise unwanted proteins away from the viral replication compartments that are sites of robust transcription, DNA synthesis, and capsid maturation. A possible role of HSPs in the baculovirus infection cycle has not been studied.

It has been demonstrated that in silkworm *Bombyx mori* cells and larvae, the switch of cellular translation machinery to polyhedrin synthesis at late stages of infection is accompanied by the inability to synthesize HSPs (Evgen'ev et al., 1989). However, the function of the heat shock pathway during virus replication and BV production has not been investigated. Whether baculoviruses activate HSR and induce synthesis of HSPs in infected cells remains unknown. These questions were addressed in this report, and experimental proof for activation of the HSR in baculovirus-infected cells was obtained. Induction of HSP/HSC70s in *Spodoptera frugiperda* (Sf-9) cells infected with AcMNPV was demonstrated by Western blotting after one- and two-dimensional PAGE fractionation of proteins from infected cells. KNK437, the inhibitor of inducible HSPs at the mRNA level, dramatically decreased vDNA synthesis and BV production suggesting an important role of HSR in baculovirus infection cycle.

Results

In order to elucidate the heat shock response (HSR) of insect cells to the baculovirus infection, the amount of HSP/HSC70 proteins in Sf-9 cells was determined at different times after AcMNPV infection using Western blot analysis. The monoclonal antibody 7.10.3 used in these experiments recognizes proteins from the HSP/HSC70 family, both constitutive and inducible, in various insect species (Garbuz et al., 2003). Two adjacent immunoreactive bands with molecular masses of about 70 kDa were revealed after SDS-PAGE fractionation of uninfected cells (Fig. 1A, lane 1). The amount of HSP/HSC70s moderately increased at 24 hpi and 48 hpi and remained at slightly elevated level up to 120 hpi (lanes 2 to 7). Progression of the virus infection was confirmed in this and other experiments by detection of the very late viral protein polyhedrin (the bottom panel in Fig. 1A). The relative changes in HSPs during infection were calculated by using β-actin as a reference (Fig. 1B). The amount of HSP/HSC70s in infected cells exceeded that in uninfected cells by 33% and 44% at 24 hpi and 48 hpi, respectively, whereas at 120 hpi it was higher by approximately 30%. These data showed that baculoviruses induce HSR and cause modest accumulation of HSP/HSC70s in infected cells.

By acting as inducers of the HSR, baculoviruses may change the sensitivity of infected cells to other environmental stresses including traditional heat shock (HS). It is difficult to predict how the first stress by viral infection modifies the reaction of cells to the second stress factor. To clarify this question, a response to heat shock was determined in Sf-9 cells infected with AcMNPV. Heating at 37 °C for 2 h increased the amount of HSP/HSC70s in uninfected cells by approximately 50% (Figs. 2A, lanes 1–2, B). However, the heating of cells infected with AcMNPV at the MOI of 10 caused a several-fold increase in the amount of cellular HSPs (Figs. 2A, lanes 3–6, B). After heating at 4 hpi and 8 hpi, the relative amount of HSP/HSC70 in infected cells was 3.5-fold and 3.8 -fold higher, respectively, than that in uninfected cells incubated at the standard conditions. The level of HSPs induction declined at the later stages of infection (22 and 46 hpi) but still exceeded the HSR in uninfected cells. Surprisingly, the prominent HSPs induction was observed at stages in infection when host protein synthesis would be shut down almost completely. When

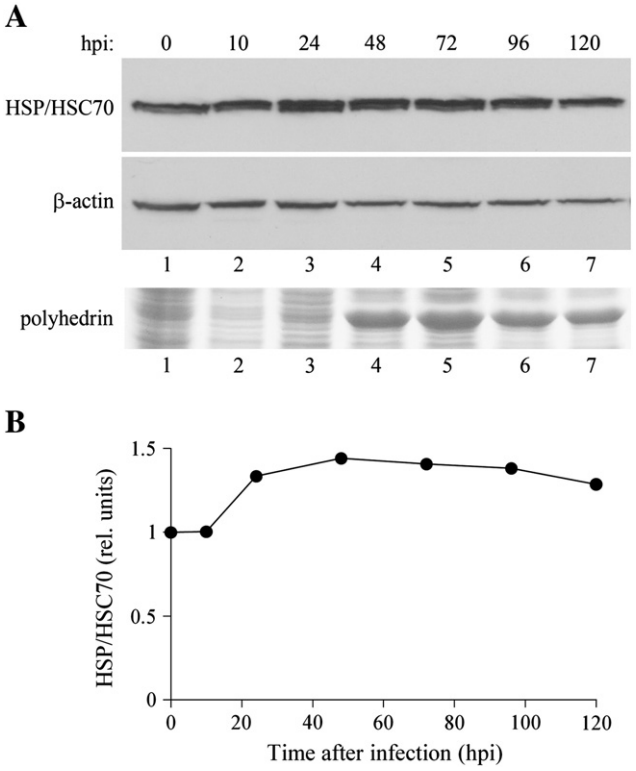

Fig. 1. Changes in the amount of HSP/HSC70s in Sf-9 cells in the course of infection with AcMNPV analyzed by Western blotting. (A) The cells were infected at the MOI of 10 and collected at indicated times. The extracts from 4×10^5 cells were fractionated by SDS-12% PAGE, blotted on membrane and probed with monoclonal antibody 7.10.3, and then with monoclonal antibody to human β-actin. Production of polyhedrin was detected by Coomassie brilliant blue staining of similar gel. Lane 1 corresponds to uninfected cells. (B) The relative amount of HSP/HSC70s normalized to the amount of β-actin in lanes of the representative gel shown in panel A. The relative amount of HSP/HSC70s in uninfected cells was taken as a unit.

the MOI during infection was lowered from 10 to 2, the induction of HSPs by heating markedly decreased. After infection at the MOI of 2, the amount of HSP/HSC70s increased by heating less than 2-fold in comparison to uninfected cells incubated at the standard conditions (Fig. 2B). A low reaction of cells infected at the MOI of 2 to the heat shock was not due to inefficient progression of virus infection. At the 46-hpi (plus 2-h heating) time point, polyhedrin appeared in cells infected at the MOI of 2 as well as in cells infected at the MOI of 10 (data not shown). The data obtained suggest that the baculovirus infection is capable of potentiating markedly HSR to elevated temperature. The virus load appeared to be a crucial factor that determines the magnitude of the reaction of infected cells to the second stress factor, HS.

More broad and diffuse bands of HSPs seen after analysis of infected and heated cells (Fig. 2A) suggest changes not only in the amount, but possibly in the variety of HSPs after virus infection and HS. To improve resolution of HSP/HSC70s that may include several constitutive and inducible species, we used two-dimensional electrophoresis with isoelectric focusing in the first direction and SDS-PAGE in the second direction followed by Western blotting with the antibody 7.10.3. Two major partially overlapping immunoreactive spots marked 1 and 2 were seen in uninfected Sf-9 cultured at the standard conditions (Fig. 3A). These species HSP(1) and HSP(2) presumably correspond to two poorly resolved bands detected in uninfected cells after one-dimensional SDS-PAGE (Figs. 1–2, lane 1). A tiny spot 3 was also observed (Fig. 3A). The spot 3 increased markedly and a new minor spot 4 appeared after heating of uninfected cells at 37 °C for 2 h (Fig. 3B) indicating induction of respective HSP(3) and HSP(4) by heat shock. The infection with AcMNPV at the MOI of 10

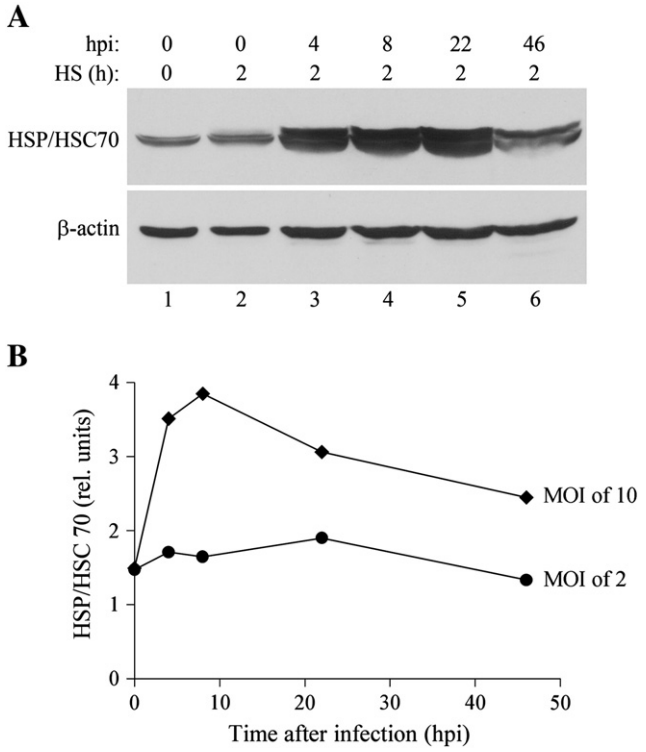

Fig. 2. Induction of HSP/HSC70s by heating of AcMNPV-infected Sf-9 cells at different times in the course of infection analyzed by SDS-10% PAGE followed by Western blotting. (A) The cells were infected at the MOI of 10. At times indicated above the panel, the cells were heated at 37 °C for 2 h (HS) and then processed as described in the legend to Fig. 1. Lanes 1 and 2 correspond to uninfected cells untreated (lane 1) or heated at 37 °C for 2 h (lane 2). (B) The relative amount of HSP/HSC70s normalized to the amount of β-actin in lanes of the representative gel shown in panel A. The results obtained with cells infected at the MOI of 2 are also shown. The relative amount of HSP/HSC70s in uninfected and untreated cells was taken as a unit.

also induced synthesis of these HSPs and appearance of one new HSP (5) (Fig. 3C). The heating of infected cells markedly increased the amount of all three inducible HSPs (3 to 5) making HSP(4) the most abundant member of HSP/HSC70 family in cells (Fig. 3D). Therefore, two-dimensional PAGE experiment allowed the identification of at least five different species of the HSP/HSC70 family in Sf-9 cells. Multiple members of the HSP/HSC70 family are known for many organisms. For example, *Saccharomyces cerevisiae* cells contain 14 members of the HSP/HSC70 family (Gong et al., 2009). Two proteins, HSP(1) and HSP(2), were expressed in Sf-9 cells cultured under standard conditions, and the amount of these HSPs did not change dramatically after HS or baculovirus infection. These species presumably represent HSPs that are expressed constitutively in Sf-9 cells. The HSPs 3 to 5 were markedly induced by HS or by virus infection, and they probably represent the inducible members of the HSP/HSC70 family. Interestingly, the heating at 37 °C for 2 h or the virus infection alone produced similar moderate HSR judging by overall accumulation of HSPs (Figs. 1 and 2). However, heating preferentially induced HSP(3) but did not induce HSP(5), whereas the virus infection induced both HSP(3) and HSP(5). HSP(5) was observed only in the infected cells. These data indicate that members of the HSP/HSC70 family in Sf-9 cells may be induced differentially by different stress factors.

The data obtained confirm that baculoviruses are capable of inducing HSR in infected cells. However, it is not clear, whether the activation of this pathway is essential for the baculovirus infection cycle. To address this question, the HSR in AcMNPV-infected Sf-9 cells was blocked by a benzylidene lactam compound KNK437. This compound prevents the development of thermotolerance in a dose-dependent manner by inhibiting the induction of various HSPs including members of the HSP/HSC70 family at the level of transcription without affecting the expression and function of constitutive forms of HSPs (Yokota et al., 2000). Incubation of uninfected Sf-9 cells with 100 μM KNK437 for 24 h caused only a decrease of approximately 10% of the cellular content of HSP/HSC70 proteins (Fig. 4, lanes 1 and 2). Infection with AcMNPV caused a moderate induction of HSP/HSC70s (lane 3) that was blocked when grown in the presence of KNK437 (lane 4). Heating of the infected cells for 2 h at 37 °C increased markedly the cellular content of HSP/HSC70s (lane 5) in agreement with data shown in Figs. 2 and 3. KNK437 strongly inhibited the induction of HSP/HSC70s in infected cells by heating, although it did not block the induction completely (lane 6). In the next experiment, we analyzed an effect of KNK437 on replication of viral DNA (vDNA) in infected cells. Sf-9 cells seeded in two six-well plastic plates were infected

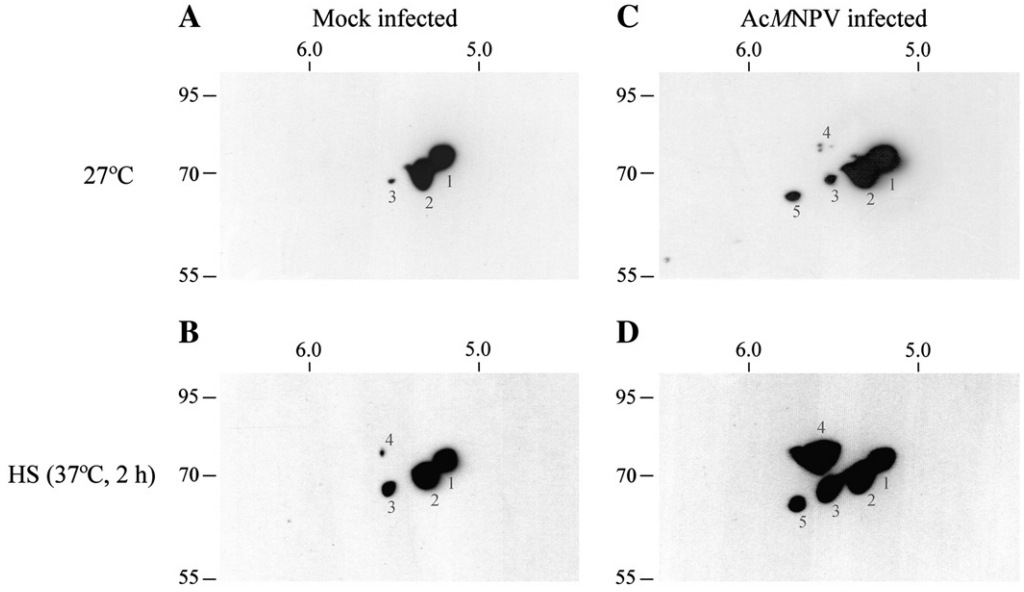

Fig. 3. Changes in the HSP/HSC70 pattern in Sf-9 cells after heating or after infection with AcMNPV analyzed by two-dimensional PAGE followed by Western blotting. Extracts were prepared from uninfected cells (A), uninfected cells heated at 37 °C for 2 h (B), cells infected with AcMNPV at the MOI of 10 and collected at 24 hpi (C), and cells infected as in panel C and heated at 37 °C for 2 h at 22 hpi (D). Electrophoresis was performed by the isoelectric focusing in the first dimension (horizontal) and then by SDS-8% PAGE in the second dimension (vertical) followed by blotting and probing with monoclonal antibody 7.10.3.

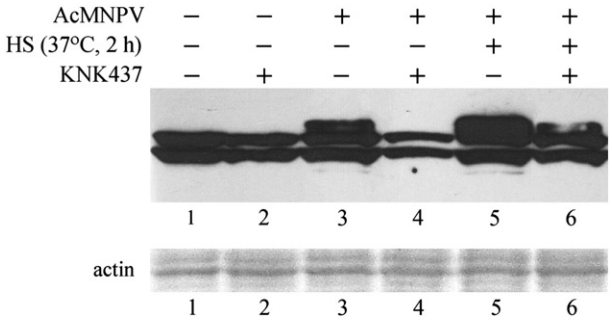

Fig. 4. Effect of KNK437 on induction of HSP/HSC70s in Sf-9 cells. Uninfected cells (lanes 1 and 2) and cells infected with AcMNPV at the MOI of 10 (lanes 3 to 6) were incubated for 24 h in the presence of 100 μM KNK437 (lanes 2, 4, 6) or in its absence (control with DMSO) (lanes 1, 3, 5). Lanes 5 and 6 show the infected cells heated at 37 °C for 2 h prior collection. Extracts were analyzed by SDS-8% PAGE followed by Western blotting. The lower part of the gel was removed and stained by Coomassie brilliant blue to visualize the actin band.

with AcMNPV at the MOI of 10, the virus was then removed, and the cells were incubated in the presence, or absence of 100 μM KNK437 (the control was DMSO). Because KNK437 was added after removal of the virus, this compound did not interfere with the virus entry into the cell. The infected cells were collected at four-hour intervals from 6 to 22 hpi and were assayed for the amount of vDNA by Real-time PCR (RT-PCR) (Fig. 5). In control infected cells, the vDNA content increased exponentially from 10 to 18 hpi with a doubling time of 1.1 h, whereas in the presence of KNK437, the average doubling time of vDNA for the same period was about two-fold lower, reaching 2.3 h. At the end of vDNA synthesis at 22 hpi, the amount of vDNA in control infected cells was 1.2 pg/cell that corresponds to approximately 10^4 copies of vDNA per cell. In infected cells treated with KNK437, the vDNA content was 20-fold lower than that in untreated cells and comprised approximately 0.06 pg/cells corresponding to 5×10^2 copies of vDNA. KNK437 did not prevent release of infectious viruses, but it markedly decreased their production. The budded virus titer in supernatants collected at 36 hpi was equal to 3.2×10^8 PFU/ml for Sf-9 cells incubated in the absence of inhibitor whereas the titer was lower by one order of magnitude in the presence of 100 μM KNK437. These data showed that the inhibition of inducible HSPs by KNK437 seriously impaired vDNA synthesis and production of budded viruses. Activation of HSR in infected cells appeared to play an important role in replication of baculoviruses.

By inhibiting HSPs, KNK437 might affect folding and trafficking of viral proteins, and their assembling into functional complexes. To clarify whether KNK437 is capable of changing subcellular localization of viral proteins, we performed confocal microscopy analysis of infected cells stained with polyclonal antibody to the viral DNA-binding protein (DBP). This protein colocalizes with other replication factors in specific nuclear foci, sites of vDNA replication, early in infection of BmN cells with BmNPV (Okano et al., 1999). In agreement with the published data for BmN cells, the nuclear fraction of DBP in Sf-9 cells was present in few nuclear speckles at the early stage in vDNA replication, 5 hpi (Fig. 6). In the presence of KNK437, DBP did not form such speckles in nucleus and showed more diffuse distribution predominantly in cytoplasm. This result suggested that KNK437 inhibits import of DBP into nucleus and its accumulation in viral replication foci. Inhibition of HSR appears to affect subcellular localization of at least one viral protein, DBP.

Discussion

The interference between baculovirus infection and HSR in insect cells is poorly understood. In this report, we showed that AcMNPV induces synthesis of HSP/HSC70s and activates HSR in Sf-9 cells. Infection by various viruses dramatically changes cellular metabolism. The baculovirus transduction of human mesenchymal stem cells perturbs the transcription of 816 genes associated with 5 signaling pathways and upregulates several signaling molecules (Chen et al., 2009). In insect cells, baculoviruses activate the mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK) and c-Jun NH2-terminal kinase (JNK), (Katsuma et al., 2007) and the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (Xiao et al., 2009). The inhibition of these signaling pathways significantly reduces virus production in infected cells. The downstream factors of the prosurvival and antiapoptotic PI3K-Akt pathway and the virally encoded inhibitors of caspases (P35 and IAPs) function presumably to prevent apoptosis triggered by viral replication factors (Schultz and Friesen, 2009). It was suggested that baculovirus replication in insect cells induces a host cell response similar to the DNA damage response in vertebrates (Schultz and Friesen, 2009), that is generally accompanied by activation of HSPs. Evidence for oxidative stress following the baculovirus infection of insect cells was previously obtained (Wang et al., 2001). In this report, the increase by approximately 40% in total amount of HSP/HSC70 proteins was observed after infection of Sf-9 cells with AcMNPV (Fig. 1). New species of HSPs were produced after infection (Fig. 3C) or heating of uninfected cells (Fig. 3B), which represent presumably the inducible members of the HSP/HSC70 family. These data suggested that baculoviruses serve as moderate inductors of HSR in cultured insect cells. However, the cellular HSP system was apparently activated by infection, and the response to HS was much stronger in infected than in uninfected cells (Fig. 2). The data obtained are of significant interest because numerous studies (for review see (Feder and Hofmann, 1999)) have demonstrated that mild HS treatment strongly increased the thermoresistance of cells or organisms to subsequent heavy heat shock challenge ("induced thermotolerance"). The virus load under infection that controls number of viral replication foci in nucleus (Okano et al., 1999) appeared to determine a quantitative response of infected cells to HS. The sensitivity to HS was developed rather early after infection, at 4 to 8 hpi, and decreased by 46 hpi (Fig. 2). Interestingly, the general shutdown of host protein synthesis at 22 hpi and 46 hpi apparently does not preclude the HSPs induction by HS. The progressive decline in HSR in the course of infections presumably causes the inability to synthesize HSPs at the terminal stages of infection cycle like was previously shown for infected silkworm cells (Evgen'ev et al., 1989). Although AcMNPV induced only moderate induction of HSPs in infected cells, the HSR was essential to sustain a high level of vDNA replication and budded virus (BV) production. In order to prevent HSR in AcMNPV-infected

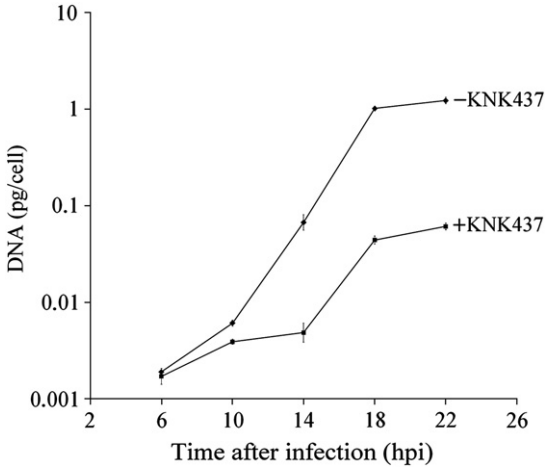

Fig. 5. vDNA synthesis in Sf-9 cells infected with AcMNPV at the MOI of 10 and then incubated in the presence of 100 μM KNK437 or in its absence. The vDNA content was determined by RT- PCR in triplicate probes for each time point. The error bars represent the standard deviation.

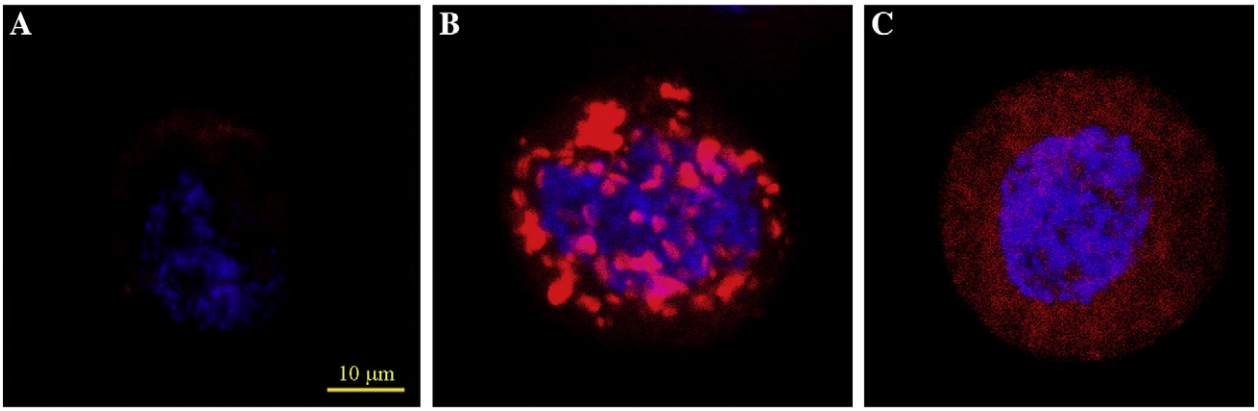

Fig. 6. Subcellular localization of DBP in Sf-9 cells infected with AcMNPV at the MOI of 10 and collected at 5 hpi. The merged images of immunostained DBP (red) and the Hoechst nuclear stain (blue) are shown for the mock-infected cells (A) and AcMNPV-infected cells incubated in the absence of inhibitor (B) or in the presence of 100 μM KNK437 (C).

cells, a benzylidene lactam compound KNK437 was used. This compound inhibits the induction of various HSPs at the level of transcription (Yokota et al., 2000). The inhibition of HSR by KNK437 reduced by more than an order of magnitude the rate of vDNA synthesis (Fig. 5) and markedly suppressed the release of infectious virions. Because the expression of late and very late viral genes depends on vDNA replication which provides templates for expression of these genes, a strong inhibitory effect of KNK437 on synthesis of late and very late viral products should be expected although this characteristic has not been studied in this report.

How HSR supports the baculovirus infection remains unknown. Multiple functions of inducible HSPs may be essential such as processing and trafficking of viral proteins and structures inside cells, protection of these proteins from unfolding and digestion by the ubiquitin-proteasome system. Interestingly, an essential replication protein of AcMNPV, the SSB LEF-3, is structurally unstable and highly sensitive to heating (Mikhailov et al., 2006). Cellular chaperones may stabilize the viral replication factors. The HSPs may participate in assembling viral replication factories and maturation of virus structures as was suggested for some other viruses (for review see (Mayer, 2005)). In the case of baculoviruses, it was indirectly confirmed by inefficient accumulation of DBP in replication foci in the presence of KNK437 (Fig. 6). Besides inducible HSPs, other factors activated by HSR might also affect the infection. It was predicted recently that heat-induced transcriptional factors are directly involved in regulation of baculovirus genes expression (Kumar et al., 2009). The complex nature of HSR in cells infected by baculoviruses and specific interaction of HSPs with viral proteins deserves further investigation.

Materials and methods

Spodoptera frugiperda Sf-9 cells were cultured in SF-900 II SFM media (Invitrogen) supplemented with 10% fetal bovine serum (FBS) in the flasks at 27 °C. The cells were infected with AcMNPV at the MOI of 10. For the heat shock, the flasks were immersed in a water bath at 37 °C for 2 h. KNK437 (*N*-formyl-3,4-methylenedioxy-benzylidene-γ-butyrolactam) obtained from Calbiochem, Inc. was dissolved in DMSO. The final concentration of DMSO in the culture medium was 0.1% (v/v).

Real-time PCR analysis of AcMNPV replication in Sf-9 cells

For measurement of viral DNA (vDNA) content in AcMNPV-infected cells by real-time PCR (RT-PCR), the R-414 kit from SYNTOL, Inc. (Moscow, Russia) and the 7500 sequence detection system from Applied Biosystems (Melbourne, Australia) were used. The forward and reverse primers designed by Rosinski et al. (Rosinski et al., 2002), respectively 5'-ATTAGCGTGGCGTGCTTTTAC-3' and 5'-GGGTCAGGCTCCTCTTTGC-3', and supplied by SYNTOL (Moscow, Russia) were used for amplification of 67-bp region in the viral DNA polymerase gene. Sf-9 cells seeded in two six-well plastic plates at density 4.5×10^6 cells/4 ml media were infected with AcMNPV at the MOI of 10, the virus was then removed, and the cells were incubated in fresh media in the presence of 100 μM KNK437 or in its absence (control with DMSO). The infected cells were collected at the four-hour intervals from 6 to 22 hpi, total DNA was purified by using the Genomic DNA purification kit (Fermentas) and dissolved in 20 μl of H_2O. The vDNA content was determined in triplicate probes of 4.5-μl in a total volume of 25 μl consisting 1 × PCR master buffer B (SYNTOL) containing the SYBR Green I fluorescent dye and the passive reference ROX, 0.25 mM each of 4 dNTPs, 2.5 mM $MgCl_2$, 300 μM each of forward and reverse primers, and 1.5 units of Tag DNA polymerase. Cycling conditions were 10 min at 95 °C, and then 40 cycles consisting of 30 s at 95 °C and 1 min at 60 °C. The 7500 Software v2.0.1. from Applied Biosystems was used for calculations. The cycle threshold (Ct) was determined at 0.1 above baseline level on exponential portion of the amplification plot ΔRn vs. Cycle. A standard calibration curve was generated from duplicate probes of purified recombinant AcMNPV DNA and serially diluted from 5 to 0.0005 ng. The cycle threshold was shown to depend on the amount of the standard viral DNA ([svDNA]) in probes (in nanograms) by following equation: $Ct = -1.44\ln[svDNA] + 18.20$, with the correlation value $R^2 = 0.999$.

Immunohistochemistry and confocal microscopy

Virus- or mock-infected Sf-9 cells were fixed for 15 min with 4% paraformaldehyde in phosphate-buffered saline (PBS), washed three times with PBS, and permeabilized for 2 min in cold acetone (−20 °C). The cells were rehydrated with PBS, blocked with 5% FBS in PBS for 1 h, and then subjected to antibody treatments. Antigen localization was determined by incubation of the cells with rabbit anti-DBP serum (Okano et al., 1999) (1:50 dilution with 5% FBS and 0.1% Triton in PBS) overnight at room temperature. After incubation with the primary antibody, cells were washed four times (5 min per wash) with PBS and then treated with the secondary antibody, Alexa-546 goat anti-rabbit IgG (1:1,000 dilution; Invitrogen) for 2 h and following incubation with Alexa- Hoechst (1:100 dilution; Invitrogen) for 20 min at room temperature. After four washes with PBS (10 min per wash), the cells were mounted with the Mowiol and were analyzed under confocal microscope Leica SPE equipped with an Ar-Kr laser. Images were recorded and processed with the Leica LCS software. To ensure equal illumination for all treatments, the same intensity and filter settings were used throughout. Images were recorded at a resolution of 1,024x1,024 pixels. Control

experiments were performed by omitting primary or secondary antibodies.

Other methods

SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described (Laemmli, 1970). Two-dimensional PAGE was carried out by method of O'Farrell (O'Farrell et al., 1977) as previously described (Garbuz et al., 2003; Ulmasov et al., 1992; Zatsepina et al., 2001). For Western blotting, proteins were transferred on Hybond-ECL membrane (Amersham) and probed with rat monoclonal antibody 7.10.3 to the HSP/HSC70 family of *Drosophila melanogaster* (Lindquist Lab.) or with mouse monoclonal antibody to β-actin (Santa Cruz). The anti-rat and anti-mouse IgG conjugated to horseradish peroxidase and the ECL detection reagents were from Amersham, Inc.

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